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The grapevine root-specific aquaporin Vv*PIP2;4N* controls root hydraulic conductance and leaf gas exchange under well watered conditions but not under water stress.

Irene Perrone[a,1,*], Giorgio Gambino[a,2], Walter Chitarra[1], Marco Vitali[1], Chiara Pagliarani[1], Nadia Riccomagno[1], Raffaella Balestrini[3], Ralf Kaldenhoff[4], Norbert Uehlein[4] Ivana Gribaudo[2], Andrea Schubert[b,1], Claudio Lovisolo[1,2]

[1] Department of Agricultural, Forestry, and Food Sciences, University of Turin, Via Leonardo da Vinci 44, 10095 Grugliasco (TO), Italy.

[2]Plant Virology Institute, National Research Council (IVV-CNR), Grugliasco unit, Via Leonardo da Vinci 44, 10095 Grugliasco (TO), Italy.

[3] Plant Protection Institute, National Research Council (IPP-CNR), Torino unit, Viale Mattioli 25, 10125 Torino, Italy

[4] Darmstadt University of Technology, Applied Plant Science, Schnittspahnstr. 10, D-64287 Darmstadt, Germany

Footnotes in the following order: financial source; present addresses of authors if different from heading; corresponding author with e-mail address.

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[*]Present address: Plant Physiology Department, Umeå University, SE-901 87 Umeå, Sweden

[b] Corresponding author: Andrea Schubert (tel +39.011.6708654; fax +39.011.6708658; e-mail andrea.schubert@unito.it)

[a] These authors equally contributed to this paper

Abstract.

We functionally characterized the grape (*Vitis vinifera* L.) *VvPIP2;4N* aquaporin gene. Expression of *VvPIP2;4N* in *Xenopus laevis* oocytes increased their swelling rate 54-fold. Northern blot and qRT-PCR analyses showed that *VvPIP2;4N* is the most expressed PIP2s gene in root. *In situ* hybridisation confirmed root localization in the cortical parenchyma and close to the endodermis.
We then constitutively overexpressed *VvPIP2;4N* in *Vitis vinifera* L. 'Brachetto' and in the resulting transgenic plants we analysed *i*) the expression of endogenous and transgenic *VvPIP2;4N* and of four other aquaporins, *ii*) whole-plant, root, and leaf ecophysiological parameters, and *iii*) leaf abscisic acid content. Expression of transgenic *VvPIP2;4N* inhibited neither the expression of the endogenous gene nor that of other *PIP* aquaporins in both root and leaf. Under well-watered conditions, transgenic plants showed higher stomatal conductance, gas exchange, and shoot growth. The expression level of *VvPIP2;4N* (endogenous + transgene) was inversely correlated to root hydraulic resistance. The leaf component of total plant hydraulic resistance was low and unaffected by overexpression of *VvPIP2;4N*. Upon water stress, the overexpression of *VvPIP2;4N* induced a surge in leaf abscisic acid content, and a decrease in stomatal conductance and leaf gas exchange. Our results show that aquaporin-mediated modifications of root hydraulics play a substantial role in the regulation of water flow in well-watered grapevine plants, while they have a minor role upon drought, probably because other signals, such as ABA, take over control of water flow.

Text.

Plant aquaporins are involved in maintaining water transport from roots to leaves and cell homeostasis under all environmental conditions (Hachez et al*.,* 2006). Reverse genetics has been up to now the most effective strategy to elucidate the physiological function of specific aquaporin genes and understand their roles in water transport and drought tolerance mechanisms in plants (Kaldenhoff et al*.,* 1998; Siefritz et al*.,* 2002). In the past decade, pioneering studies of overexpression or silencing of aquaporin genes have been carried out in herbaceous model plants (reviewed in Kaldenhoff et al., 2008). To date, with the exception of transgenic *Eucalyptus* overexpressing a radish *PIP* aquaporin (Tsuchihira et al*.,* 2010), reverse genetics studies have been performed in herbaceous species such as *Arabidopsis* (Cui et al., 2008; Peng et al., 2008; Postaire et al., 2010), *Oryza sativa* (Li et al., 2008; Matsumoto et al., 2009), *Solanum lycopersicum* (Sade et al*.,* 2009), and *Nicotiana tabacum* (Zhang et al., 2008). These plants, however, are not well suited for the task of assessing the role of aquaporins in complex processes of water transport and homeostasis, such as the transduction of hydraulic and non-hydraulic messages (Lovisolo et al., 2010) and the formation and recovery of embolisms (Secchi and Zwieniecki, 2010), which are typical of woody plants. Understanding the role of aquaporins in these processes requires reverse genetics to be applied to woody plants, despite the greater difficulty generally encountered in efficiently delivering foreign constructs into their DNA.

Many grapevine (*Vitis ssp.*) species are well adapted to drought conditions and are thus expected to have developed efficient mechanisms to transfer water to their growing aerial organs and to limit negative effects of drought. Patterns of aquaporin expression and function are suspected to contribute significantly to this adaptation (Galmès et al., 2007; Vandeleur et al., 2009). Cultivated grapevine (*Vitis vinifera* L.) is traditionally a non-irrigated crop, and both yield and berry quality strongly depend on the vine's adaptability to drought (molecular and physiological aspects of grapevine drought responses are reviewed in Lovisolo et al., 2010).

For years, protocols for genetic transformation of grapevine have not been standardized into routinely applicable methods. Nevertheless, significant advances have been recently made in the development of transgenic technology, and transgenic plants from commercially important cultivars have been produced (Reustle and Büchholz, 2009). However, genetic transformation of grapevine has been mainly focused on enhancing disease resistance against pathogens, and only a few studies aimed at elucidating the functions of endogenous genes have been carried out to date (Tesnière et al., 2006; Cutanda-Perez et al., 2009).

Eighty-three sequences annotated as aquaporins, belonging to different *Vitis spp*., are present in GenBank, and 28 putative aquaporin genes have been identified in the *V. vinifera* genome (Fouquet et al*.,* 2008; Shelden et al*.,* 2009). Expression of some of these genes, belonging to the PIP (Plasma membrane Intrinsic Proteins) and TIP (Tonoplast Intrinsic Protein) subfamilies, has been studied in grape berry (Picaud et al., 2003; Fouquet et al., 2008; Choat et al., 2009) and vegetative tissues (Baiges et al*.,* 2001; Vandeleur et al*.,* 2009). The results of these studies suggest that aquaporins have important roles in facilitating water redistribution within the growing berry (Picaud et al., 2003; Fouquet et al., 2008; Choat et al., 2009), and in regulating adaptation to water stress (Vandeleur et al*.,* 2009). However, these results, being based on correlations between expression of aquaporin genes and morphological and physiological observations, lack reliable *in vivo* functional evidence.

In the present study, we adopted a reverse genetics approach to elucidate the role of a PIP-type aquaporin gene in grapevine. We constitutively overexpressed *VvPIP2;4N* in *V. vinifera* plants. Physiological and molecular parameters of six independent transgenic lines were analysed in well-watered and drought conditions. The expression level of endogenous *VvPIP2;*4*N* and of other aquaporins was assessed in order to investigate possible compensation effects present within the gene family. For each line we analysed the single resistances that control water flow across the plant. The behaviour of transgenic lines with a different level of transgene expression allowed us to correlate gene expression to physiological parameters and to hypothesize specific functions of this aquaporin.

RESULTS

Cloning and *in silico* analysis of *VvPIP2;4N*

A putative aquaporin gene was isolated from cDNA of *V. vinifera* cv Nebbiolo using primers designed on an EST contig sequence. The isolated gene (DQ358107) resulted nearly identical in its coding sequence (single nucleotide mismatch, not leading to aminoacid change) to the gene predicted in the grapevine (cv PN40024) 12X genome draft (http://genomes.cribi.unipd.it/) at locus VIT06s0004g02850 on chromosome six, and called *VvPIP2;4* by Shelden et al. (2009). The same authors isolated an aquaporin sequence they called *VvPIP2;4* (EF364438) from the cv Cabernet Sauvignon. However, this sequence shares a mere 83% identity at the aminoacidic level with the

gene we isolated, and is nearly identical (five nucleotide and one aminoacid mismatches) to *VvPIP2;1,* described by Fouquet et al. (2008; DQ834698) and later by the same authors (Shelden et al*.,* 2009; AY823263) (Fig. 1). Given that functional characterisation of the *VvPIP2;4* gene at the water transport (Shelden et al*.,* 2009) and expression pattern levels (Vandeleur et al*.,* 2009) was performed using GenBank accession EF364438, our gene represents a yet uncharacterised PIP2 from *Vitis vinifera* L.. To avoid confusion, we have used the name *VvPIP2;4N* for the gene we isolated from 'Nebbiolo'.

The transcript sequence (843 bp) of *VvPIP2;4N* encodes a protein that is 280 aminoacids long, with theoretical pI and Mw of 7.00 and 30 kDa respectively, similarly to PIP2s from other species. Analysis of the deduced protein sequence of *VvPIP2;4N* allowed the prediction of all residues characteristic of the PIP family: six transmembrane domains connected by five loops, two NPA motifs, which are conserved features of all aquaporins (Chrispeels and Maurel, 1994), and the sequences G-G-G-A-N-X-X-X-X-G-Y in loop C and T-G-I/T-N-P-A-R-S-L/F-G-A-A-I/V-I/V-F/Y-N in loop E, (Barone et al*.,* 1997). VvPIP2;4N also shows a shorter N-terminal region and a longer C-terminal region when compared to PIP1 proteins, as described in literature (Chaumont et al*.,* 2000). Residues known to control protein activity are present, such as His 194, located in loop D and involved in pH sensing (Tournaire-Roux et al*.,* 2003), as well as two putative phosphorylation sites, Ser 116 and Ser 275, present in loop B and in the C-terminal region (Johansson et al*.,* 1998), and the diacidic DVE motif (Asp-Val-Glu), which has a putative role in endoplasmic reticulum export (Zelazny et al., 2009; Sorieul et al., 2011) (Fig. S1).

Functional characterisation of *VvPIP2;4N*

Xenopus oocytes expressing *VvPIP2;4N* were exposed to osmotic shock. The expression of this aquaporin triggered a rapid increase in oocyte volume, with a P_f value of 0,067 cm s^{-1}, meaning a 54-fold increase in the swelling rate when compared with control oocytes. Addition of $HgCl_2$ to the incubation solution caused an approximately 2-fold decrease in osmotic permeability, showing an inhibition by mercury common to most plant aquaporins (Hukin et al., 2002) (Fig. 2) and comparable to what observed for OePIP2;1 in the same experimental conditions (Secchi et al., 2007). Higher reductions of Pf have been obtained with mercury treatment of aquaporin expressing oocytes, but at higher doses, e.g. 1 mM for 30 min for NtPIP2;1 (Biela et al., 1999) and 0.5 mM for *Samanea saman* SsAQP2 (Moshelion et al., 2002).

Transcript concentration analysis by Northern blot was performed in different 'Nebbiolo' organs, using the full length *VvPIP2;4N* probe. Despite the high homology between PIP2 genes, a single band was detected in a Southern blot hybridization of 'Nebbiolo' genomic DNA with the same probe, indicating that it hybridized to a single locus (Figure S2C) and supporting its specificity within the PIP2 subfamily. Strong expression of *VvPIP2;4N* was observed in roots, whereas transcripts were barely present in shoots and leaves (Fig. 3A). These results are in contrast with the very low expression levels of *VvPIP2;4* in roots reported by Vandeleur et al. (2009); however no correspondence was observed between the primers used by those authors and those used in this study, confirming that *VvPIP2;4N* is different from *VvPIP2;4* described by Shelden et al. (2009) and Vandeleur et al. (2009). The expression pattern of *VvPIP2;4N* suggests that this aquaporin could be a specific root isoform, probably involved in the regulation of root hydraulic conductance.

In order to localize *VvPIP2;4N* transcripts, *in situ* hybridization experiments were carried out in different tissues of 'Nebbiolo' plants: roots, stems and leaves. Results confirmed that expression was concentrated in roots, while only a faint signal was observed in leaves, and no signal at all was detected in shoots. *VvPIP2;4N* expression was widespread in the root elongating and absorbing apical zones, and transcripts were observed in the cortex and associated with pericycle and vascular bundles (Fig. 3, B-D). In the older parts of roots (more than 3 mm distance from the apex) *VvPIP2;4N* mRNA became restricted to the xylem bundles (Fig. 3, E and F). Control experiments with the sense probe did not show a significant signal (Fig. 3, G-M), while a high chromogenic signal became evident when using a ribosomal antisense 18S probe as positive internal control (Fig. 3N-Q). By staining with 0.1% berberine we observed a progressive suberification of tangential cell walls with the formation of an exodermis and of an endodermis, showing evident Casparian bands, starting at about 2 mm from the apex (Fig. 3, R-U).

We then analysed by means of qRT-PCR the expression pattern of *VvPIP2;4N*, together with other known PIP2 genes and a PIP1-type aquaporin, in the Brachetto cultivar, which was subsequently used for transformation experiments. Results confirmed a root-specific expression pattern of *VvPIP2;4N*. In roots of well-watered plants, *VvPIP2;4N* was the most abundant PIP2-type aquaporin transcript, followed by *VvPIP2;2* and *VvPIP1;1*, which were present in roots but virtually absent in leaves. In 'Brachetto' we also tested the effects of water stress, which induced downregulation of *VvPIP2;4N* in roots and in leaves. Accordingly, the PIP2-type aquaporin with the highest expression under these conditions was *VvPIP2;2* in both organs (Fig. 4A).

Transgenic grapevine plants

To investigate the role of *VvPIP2;4N in vivo*, we ectopically expressed *VvPIP2;4N* in 'Brachetto' plants using a CaMV 35S promoter. Thirty-three putatively transgenic grapevine lines transformed with pJam1469-35s::*VvPIP2;4N* binary vector, showing transgene products of the expected size for both *npt*II and the adjacent *VvPIP2;4N* genes, were obtained by regeneration and micropropagation. About 85% of transgenic lines displayed only one or two T-DNA insertions. Several lines showed the same hybridization pattern (Fig. S2, B and C). The 33 regenerated transgenic lines of 'Brachetto' arose from 11 independent transformation events, as grouped according to Southern blot results. Six lines with different T-DNA configurations were chosen for further molecular and physiological analyses (Tab. S1).

Within the selected lines, transgenic *VvPIP2;4N* expression was lowest in line 23 in both roots and leaves, and highest in lines 16 in roots and in 24 and 33 in leaves. The expression of the transgene was higher than in roots in four lines (23, 24, 28, and 33); we observed an expression decrease in line 28, and an increase in line 4, compared to well-watered conditions (Fig. 4B).

In order to check for modifications in the transcription of endogenous genes (Heinen et al*.,* 2009), we assessed the expression level of endogenous *VvPIP2;4N*, and of four other PIP genes, in the transgenic lines. The concentration of endogenous *VvPIP2;4N* transcripts was mostly unaffected by the presence of the transgene in the roots of well-watered plants, while, under water stress, transgenic plants showed an increase in the expression of endogenous *VvPIP2;4N*. Expression of *VvPIP1;1, VvPIP2;1,* and *VvPIP2;2* in transgenic plants was in some cases higher than in wild-type plants (e.g. *VvPIP2;1* in lines 4 and 28) (Fig. S3).

Effects of *VvPIP2;4N* overexpression on hydraulic conductivity and leaf gas exchange

The availability of six different transgenic grapevine lines with different *VvPIP2;4N* transcript levels allowed us to study the correlation between expression of this aquaporin and water transport processes at the whole-plant level.

Under well-watered conditions, most transgenic lines showed higher stomatal conductance (g_s) (Fig. 5A) than wild-type plants, and g_s was positively correlated to root *VvPIP2;4N* transcript levels. Similarly, leaf transpiration (E) and net photosynthesis (P_n) were higher in transgenic plants and were correlated to the level of transgene expression (Fig. S4, A and B). Leaf water potential was lower in some transgenic lines (4, 16, and 33) than in wild-types (Fig. 5B).

Upon water stress, transgenic plants showed a slight decrease in g_s and leaf gas exchange compared to wild-type plants, while water potential was not affected. (Fig. 5, A and B; and S4, A and B). Similar observations were made for both irrigated and water-stressed plants when *VvPIP2;4N* expression in leaves was addressed (Fig. 5, D and E).

Although net photosynthesis increased in irrigated transgenic plants, water use efficiency (WUE) and concentration of CO_2 in intercellular spaces (C_i) did not significantly change between transgenic and wild-type grapevines. This suggests that *VvPIP2;4N* overexpression did not affect the photosynthetic machinery, while the increase in photosynthesis was rather due to increased stomatal conductance. Water stress impaired WUE and enhanced Ci, but showed no divergent effects on wild-type and transformed plants (Fig. S4, C and D).

In irrigated plants, ABA concentration was low, and was not affected by transgene expression. Leaf ABA concentration was significantly higher in water-stressed than in irrigated plants of all lines, and in water-stressed plants it increased significantly in transgenic plants, alongside the increase in *VvPIP2;4N* expression (Figure 5, C and F).

Shoot weight of well-watered plants showed a tendency to increase (although not significantly) with increasing *VvPIP2;4N* expression, while root weight remained stable (Figure 6). Whole-root hydraulic resistance ($R_{h\ root}$,) and surface area-specific root hydraulic resistance ($R_{s\ root}$), measured with the high pressure flux method (HPFM), were lower in most transgenic plants than in wild-type ones, and were negatively correlated to the expression of *VvPIP2;4N* (endogenous + transgene) in roots (Fig. 7A). By directly measuring soil and shoot water potential, we also assessed $R_{h\ root}$ by using the evaporative flux method (EFM). Results were in agreement with those obtained with the HPFM, although EFM values were slightly higher than HPFM values. The EFM, differently from the HPFM, could be used to assess $R_{h\ root}$ upon conditions of water stress: results again showed a negative dependency on the level of transgene expression (Fig. 7B).

Using the EFM we partitioned, in well-watered conditions, the hydraulic resistance of the whole plant ($R_{h\ plant}$), obtaining leaf hydraulic resistance ($R_{h\ leaves}$). $R_{h\ plant}$ was significantly lower in all transgenic lines. $R_{h\ leaves}$ did not display any changes between wild-type and transgenic plants, showing that changes in $R_{h\ plant}$ were substantially due to modifications of the $R_{h\ root}$ induced by transgene expression (Fig. 8).

DISCUSSION

Isolation and functional characterisation of *VvPIP2;4N*

PIP2s are considered the major facilitators of transmembrane water transport in plants (Kaldenhoff and Fischer, 2006). They increase swelling of *Xenopus* oocytes in hypotonic solutions (Chaumont et al*.,* 2000), and enhance cell membrane water permeability and whole-tissue water conductivity in *Arabidopsis* (Martre et al*.,* 2002) and rice (Katsuhara et al*.,* 2003). In grape, partly because of the existence of different genotypes, the composition of the PIP2 subfamily has not yet been fully defined. Fouquet et al. (2008) proposed four members for this subfamily in the 'PN40024' homozygous, fully sequenced genome, naming them from *PIP2;1* to *PIP2;4*. Later on, four *PIP2s,* named from *PIP2;1* to *PIP2;4,* were isolated from the heterozygous 'Cabernet Sauvignon' cultivar (Shelden et al*.,* 2009): however, while *PIP2;1* to *PIP2;3* correspond to the PN0024 genes, *VvPIP2;4* of Cabernet Sauvignon appears to be an allele of *VvPIP2;1* from 'PN40024'. The size of the PIP2 subfamily is reduced in grape when compared to other plants: eight and 11 members are present respectively in *Arabidopsis* (Johanson et al*.,* 2001) and in rice (Sakurai et al*.,* 2005).
In this study, we have cloned, from the Nebbiolo cultivar, the gene homologous (100% aminoacid identity) to *VvPIP2;4* from 'PN40024'. The sequence of this gene (which we refer to as *VvPIP2;4N*) only shares an 83% aminoacid identity with *VvPIP2;4* isolated by Shelden et al. (2009) in 'Cabernet Sauvignon', thus appearing to be an uncharacterised grape PIP2 gene. Functional assays in oocytes showed that *VvPIP2;4N* expression induced a 54-fold increase in membrane osmotic permeability (P_f), a value comparable to those detected when using this experimental system for plant aquaporins (Marjanovic et al., 2005; Secchi et al., 2007) and to those reported for the four grapevine PIP2 aquaporins analysed by Shelden et al. (2009). Taken together, these results suggest that *VvPIP2;4N* is a water-transporting aquaporin which may potentially offer a remarkable contribution to water transcellular transport in grape tissues.

VvPIP2;4N is a root-specific grapevine aquaporin

Several pieces of evidence support the idea that specific aquaporins may have specific functional roles different from those played by their relatives belonging to the MIP family. For example, in *Arabidopsis,* facilitation of CO_2 transport has been demonstrated for *AtPIP1;2* but not for *AtPIP2;3* (Heckwolf et al*.,* 2011). Tissue localization patterns can be used as indicators of functional

specificity, since physiological roles can be restricted to specific tissues and organs. Our results in 'Brachetto' show that *VvPIP2;1* and *VvPIP2;2* are expressed in both roots and leaves, while *VvPIP2;3* is almost absent in both organs, thereby confirming previous reports (Baiges et al*.,* 2001; Vandeleur et al*.,* 2009). However, among the PIP2 genes, *VvPIP2;4N* shows a characteristic root-specific expression profile, resembling other root-specific aquaporins, such as rice *OsPIP2;5* (Sakurai et al*.,* 2005). *In situ* hybridization shows that *VvPIP2;4N* expression is localized in the cortex and associated to the vascular bundle of young roots, where an endodermis is developing, thus confirming the root tissue localization pattern of other *PIP* transcripts, such as grapevine *VvPIP2;2* (Vandeleur et al., 2009) and tobacco *NtPIP1;2* (Otto et al*.*, 2000).

The root localization of *VvPIP2;4N* agrees with its putative function in the control of transmembrane water flow in the root. Expression of this aquaporin is higher in most transgenic lines than in wild-type plants, and is correlated to root hydraulic conductance. In wild-type plants, *VvPIP2;4N* is the only PIP2 downregulated by water stress, and root hydraulic conductance is affected accordingly. Correlations between the expression of specific aquaporins in roots and root hydraulic conductance have been reported several times, especially in plants where suberified exodermis and endodermis develop (Barrowclough et al*.,* 2000; North et al*.,* 2004), as is the case for grapevine. Subsequently, our results suggest that *VvPIP2;4N* may act as a major controller of root hydraulic conductance in grapevine plants.

Transgenic grapevine plants overexpressing *VvPIP2;4N*

Transgenic grapevines containing the *VvPIP2;4N* transgene were obtained from embryogenic cultures of 'Brachetto'. Although transgenic plants of 'Nebbiolo' have previously been obtained (Gambino et al., 2005), we have chosen the 'Brachetto' variety for its high capacity to induce somatic embryogenesis and plant regeneration after genetic transformation (Gambino and Gribaudo, unpublished data). Furthermore, both genotypes show an anisohydric behaviour when subjected to water stress (Fig. S5). The hybridization patterns observed for the different plants in Southern analyses followed a few common motifs, which allowed the clustering of the transgenic lines into groups, whose members presumably issued from the same transformation event. A similar situation has been previously reported in other grape transformation experiments (Iocco et al., 2001; Gambino et al., 2005; Maghuly et al., 2006). The transformation protocol we used requires maintenance of embryogenic cultures, after *Agrobacterium* co-culture, for several months under kanamycin selection pressure before embryo germination. Although this protocol favours the

recovery of genetically uniform transgenic plants without escapes, it has the tendency to induce the proliferation of numerous embryos from the same transformed cellular clone, thus originating several plants deriving from a single transformation event.

The insertion of foreign DNA into a plant genome can lead to alterations in its structure, which can affect transgene expression (Gelvin, 2003). Coherently, analysis of transgene expression by qRT-PCR revealed important differences among transformed lines, and a lack of correlation between T-DNA copy numbers and transgene-derived mRNA accumulation.

Although *VvPIP2;4N* was under control of the constitutive 35S promoter, its expression varied from organ to organ and from well-watered conditions to drought. Such results have been rarely reported in herbaceous transgenic plants, where transgenic lines are stabilised after several cycles of self-crossing. However, these results are not surprising in woody plants, where hemizygous T0 transgenic plants are commonly employed (Kumar and Fladung, 2001), and where developmental stages and environmental conditions can affect the expression of transgenes introduced under a constitutive promoter. D'Angeli and Altamura (2007), for instance, showed that a transgenic protein was detected in olive stems but not in leaves and meristems. In grapevine, transgene expression decreases following transfer from *in vitro* culture to greenhouse, concomitantly to an increase in symmetric cytosine (CpG) methylation in the transgene sequence (Gambino et al*.,* 2010). Epigenetic phenomena such as DNA methylation or histone modification could be the causes of the observed variations in transgene expression. It must be furthermore noticed that aquaporin activity is also controlled by post-transcriptional regulation processes, such as phosphorylation, interaction with protons or with inhibitors such as H_2O_2 (Maurel et al., 2008). However, several reports have confirmed that significant relationships between aquaporin expression and water transport processes can be observed in plants, independently of possible post-transcriptional regulation effects (Hachez et al., 2006; Kaldenhoff et al., 2008).

VvPIP2;4N overexpression enhances water transport at the whole-plant level underwell-watered consitions but not upon drought

Transgenic grapevines overexpressing *VvPIP2;4N* showed increased stomatal conductance, leaf gas exchange and shoot growth rates in well watered conditions compared to wild-type controls. These results are in agreement with the conclusions drawn by several studies concerning the effects of PIP2 overexpression in tobacco (Aharon et al*.,* 2003), rice (Katsuhara et al*.,* 2003), and *Eucalyptus* (Tsuchihira et al*.,* 2010). These effects seemed to be guided by the increase in stomatal

conductance, although none of the major known regulators of stomatal conductance in grape (ABA, subcellular CO_2 concentration, and leaf water potential) were substantially modified by transgene expression under favourable conditions (leaf water potential in some transgenic lines actually decreased, which would involve stomatal closure). *VvPIP2;4N* transcripts are absent in guard cells (as in the whole leaf) in wild-type plants, while the gene is expressed in the leaves of transgenic plants. It has been shown that the 35S promoter efficiently drives expression in Arabidopsis guard cells, although at a lower level than in other tissues (Yang et al., 2008). Thus we hypothesize that in transgenic plants stomatal conductance may directly and positively be affected by the increased turgor of guard cells brought by the constitutive expression of *VvPIP2;4N* in their plasma membrane. Under well-watered conditions, with low leaf water potential and low ABA levels, guard cells actively build up turgor, and overexpression of an aquaporin could facilitate water uptake from the intercellular spaces, thereby enhancing stomatal opening.

Although the benefits brought by *VvPIP2;4N* overexpression to leaf gas exchange and shoot growth in well-watered conditions have been proven here, the situation concerning water-stressed grapevines was quite different. Herbaceous species overexpressing aquaporins showed reduced resistance to drought stress compared to wild-type plants, paired with the acceleration of wilting (Aharon et al*.,* 2003; Katsuhara et al*.,* 2003). By contrast, in our transgenic grapevines, *VvPIP2;4N* overexpression did not induce any signs of wilting. In addition, water potential measurement indicated that water stress conditions were not more severe than those applied to wild-type plants, similarly to what has been reported for *Eucalyptus* overexpressing a PIP2 (Tsuchihira et al*.,* 2010). These results suggest that in grape (and possibly other plants), under water stress, mechanisms other than the hydraulic conductance of cell membranes are responsible for water flow across the plant and into the environment.

We provide evidence that one such mechanism is leaf ABA concentration, which was unaffected by transgene expression when plants were irrigated, but was positively correlated to *VvPIP2;4N* expression in water-stressed grapevines. In grapevine, the control of ABA on drought response is crucial, and this aspect has been well characterized in literature (Loveys, 1984; Lovisolo et al., 2008a). In our plants, under well-watered conditions, leaf ABA concentration was not correlated to transpiration, while upon water stress a higher ABA concentration in transgenic plants corresponded to a lower stomatal conductance compared to the (already low) values observed in wild-type plants. Upon the sensing of water stress in roots, in grapevine ABA is transported from the roots to the leaves via the xylem (Stoll et al., 2000). Although we have no experimental supporting data, we hypothesize that *VvPIP2;4N* overexpression in roots, by increasing root

hydraulic conductance, could induce water loss to the soil, and as a consequence a more severe stress than in roots of wild-type plants and a more intense mobilization of ABA toward the leaves. This mobilization could, in turn, effectively safeguard tissues from wilting, and maintain control of water flow at the stomatal level.

Leaf hydraulic resistance is not affected by *VvPIP2;4N* overexpression

Although *VvPIP2;4N* overexpression effectively modified root hydraulic conductance, partitioning of plant hydraulic resistance at the organ level showed that leaf resistance was not affected, despite the fact that the transgene was expressed in both roots and shoots. Leaf hydraulic resistance is controlled by cell-to-cell water movement at different degrees in different species (Tyree *et al.,* 2005). Increases in leaf conductance induced by light exposure coincide with enhancement of aquaporin expression in walnut (Cochard et al*.,* 2007). Accordingly, overexpression of aquaporin genes increased rosette conductivity in *Arabidopsis* (Postaire et al*.,* 2010) and leaf conductivity in rice (Li et al*.,* 2008). Nardini et al. (2005) showed that in grapevine, as in other sun-adapted species, the contribution of mesophyll hydraulic resistance to total leaf resistance is relatively high, and overexpression of an aquaporin in the leaf should lead to a reduction of leaf resistance. However, the technique of minor vein cutting used by these authors includes within the concept of mesophyll resistance only the resistance opposed to flow after water exits the apical part of minor (fifth order) veins, while water can also exit the vasculature by directly crossing the bundle sheath cells surrounding the proximal part of minor veins and lower-order veins. Grapevine has heterobaric leaves showing bundle sheath extensions (BSEs) that create transparent, non photosynthetic regions on the leaf lamina (Liakoura et al*.,* 2009). Recently, Buckley et al. (2011) showed that, in heterobaric leaves, BSEs reduce hydraulic resistance between bundle sheath and epidermis cells (r_{be}). A further reduction of the resistance along this pathway, that could be obtained by overexpression of an aquaporin, would not be expected to significantly affect leaf resistance, in opposition to what could happen in a homobaric leaf (a leaf without BSEs), where r_{be}, is one order of magnitude higher than in heterobaric ones (Buckley et al*.,* 2011).

Another possible explanation of the apparent inactivity of transgenic VvPIP2;4N in leaves is based on the possibility that, *in vivo,* its activity might require interaction with other proteins. According to the observations made by Vandeleur et al. (2009), grapevine *VvPIP2;2* requires co.expression of *VvPIP1;1,* to be fully active as a water channel. A similar interaction of PIP1s with PIP2s was previously reported (Fetter et al., 2004) and can be due to heteromerization of the aquaporin

tetramer (Otto et al., 2011). We observed that *VvPIP1;1* was not expressed in leaves, confirming the results of Baiges et al. (2001), while it was expressed in roots, in particular in some transgenic lines (4, 28, 33) characterized by low R_h. Thus, although VvPIP2;4N efficiently facilitates transmembrane water transport when expressed alone in oocytes, the possibility exists that it may require interaction with a PIP1 such as VvPIP1;1 to be fully active *in planta* as a water transport facilitator.

CONCLUSION

In this study we have characterized a root-specific aquaporin of grapevine, and we have overexpressed it in order to provide information about its functional properties. VvPIP2;4N acts as an effective aquaporin in oocytes, and its overexpression affects leaf gas exchange and root hydraulic conductance, but not leaf hydraulic resistance, under irrigated conditions. Under water stress conditions, however, the potentially negative effects of overexpression of this aquaporin are avoided by ABA control of stomatal opening. As grapevines worldwide are grafted, the existence of this aquaporin in commercial grapevine rootstocks could potentially represent a marker of vegetative growth in vineyards where adequate irrigation or rainfall is available.

MATERIALS AND METHODS

Bioinformatic analyses and cDNA cloning of *VvPIP2;4N*

Total RNA was isolated from roots of *V. vinifera* L. cv Nebbiolo. First-strand cDNA was synthesized from total RNA treated with DNaseI (Invitrogen, http//www.invitrogen.com/) using oligo-$(dT)_{12–18}$ as primers and SuperScript II Reverse Transcriptase (Invitrogen). Gene-specific primers (Table S1) were designed based on a TC sequence (TC38138) deposited at the *Vitis vinifera* Gene Index (release 4.0) database (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=grape/). PCR was performed using Platinum Pfx DNA Polymerase (Invitrogen), according to the manufacturer's instructions. The expected product length was gel-purified, inserted into the pGEM-T Easy vector (Promega, www.promega.com) and sequenced

using M13 forward and reverse primers. The isolated sequence was registered in Genbank with the accession number DQ358107 and was given the name *VvPIP2;4N*.
The protein sequence was deduced by applying the ExPASy Translate tool (http:// expasy.org/tools/dna.html) and the theoretical pI and Mw were calculated using the ExPASy Compute pI/Mw tool (http://expasy.org/cgi-bin/pi_tool/). Prediction of transmembrane domains of the deduced protein sequence was performed by the TMHMM software (http://www.cbs.dtu.dk/services/TMHMM/).

Functional analysis in *Xenopus laevis* oocytes

The function of *VvPIP2;4N* gene was analysed in *X. laevis* oocytes following the method described by Biela et al. (1999). Fifty ng of cRNA (or an equivalent volume of water) were injected per oocyte and, 3 days after cRNA injection, the osmotic water permeability coefficient (P_f) was determined by measuring the rate of oocyte swelling induced by a hypo-osmotic shock of 160 mOsm kg^{-1}. The P_f was calculated using the formula: $P_f = V_0[d(V/V_0)/ dt]/[S*V_w(Osm_{in} - Osm_{out})]$, where the initial oocyte volume (V_0) and the initial oocyte surface area (S) were calculated from every single oocyte 5 sec after transferring them into hypotonic medium. The molar volume of water (V_w) is given as 18 cm^3 mol^{-1}.

Expression profile of *VvPIP2;4N*

Samples for expression analysis were collected at 11.00 am to avoid interference from diurnal patterns of aquaporin expression. Total RNA was isolated from roots, stems and leaves of 2-year-old 'Nebbiolo' plants grown under greenhouse conditions. Northern hybridizations were carried out using a specific DNA probe corresponding to the full length *VvPIP2;4N* sequence. The probe was amplified by PCR using the primers reported in Table S2, and digoxigenin labelled, using the PCR DIG Probe Synthesis Kit (Roche, http//www.roche.com/) according to manufacturer's instructions. Blots were stripped and reprobed with 18S rDNA probes.
In situ hybridization was performed using *Vitis vinifera* roots apexes and young leaves. Tissue was fixed in 4% paraformaldehyde overnight at 4 °C, washed with saline solution (150 mM NaCl), dehydrated in a graded ethanol series, cleared in Bio-Clear (Bio-Optica, http//www.bio-optica.it/) and infiltrated with Paraplast plus (Sigma, http//www.sigmaaldrich.com/). Longitudinal and transversal sections of 8 μm were transferred to poly-L-lysine (Sigma) pre-treated slides and dried

overnight at 40 °C. RNA hybridization and detection experiments were carried out on dewaxed sections as described (Balestrini et al., 2000), using DIG-labelled RNA probes. Sense and anti-sense probes were generated by *in vitro* transcription of linearized template DNAs as described by the manufacturer (Roche). After hybridization and detection, the sections were dehydrated through an ethanol-Bio-Clear series and mounted in Bio-Mount (Bio-Optica). The berberine-aniline blue fluorescent staining method (Brundrett et al*.,* 1988) was applied to root sections to detect the presence of suberin. After treatment, sections were observed under UV light with a Leitz Ortholux (http//www.leitz.com/) microscope.

Grapevine transformation and selection

The *VvPIP2;4N* gene was cloned into pJam1469 binary vector, obtained by insertion of a CaMV 35S promoter cassette (http://www.pgreen.ac.uk/a_cst_fr.htm) into the pBIN19 plasmid (Bevan, 1984). The vector harbouring the *VvPIP2;4N* gene (pJam1469-35s::*VvPIP2;4N*) was inserted into *Agrobacterium tumefaciens* LBA4404 strain through heat-shock transformation.

Embryogenic calli of *V. vinifera* cv Brachetto were transformed following the protocol of Gambino et al*.* (2005). Single embryo-derived plantlets were micropropagated by repeated subcultural apical cuttings on MS medium, without plant growth regulators and with kanamycin. The plants were acclimatized and transferred to a greenhouse. Expression and physiological analyses of the plants were carried out during the following summer.

PCR and Southern blotting confirmed transformation. DNA was extracted from grapevine plantlets grown *in vitro*, using the method of Thomas et al. (1993). PCR amplification was performed using specific primer pairs for transgenic *VvPIP2;4N* (Table S2) and *nptII* genes (Gambino et al., 2010). For Southern hybridization, genomic DNA was digested by means of restriction endonucleases *Hind*III and *Eco*RI (50 units each; Promega), and *nptII* gene was used as a DIG-labelled probe. To exclude cross hybridization between *PIP*s genes, we performed a Southern analysis on 'Nebbiolo' genomic DNA digested by *Xba*I restriction endonuclease (Promega) and hybridized with the full length *VvPIP2;4N* DIG-labelled probe, used for Northern and *in situ* hybridizations.

Expression analyses of aquaporin in transgenic grapevines

Primers for the endogenous and transgenic *VvPIP2;4N* sequences were designed using Primer Express 3.0 software (Applied Biosystems, http//www.appliedbiosystems.com/), while primers

previously reported by Choat et al. (2009) were used to amplify *VvPIP2;1*, *VvPIP2;2, VvPIP2;3* and *VvPIP1;1* (Table S2). First-strand cDNA synthesis was performed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems), and PCR reactions were performed using PowerSYBR Green master mix (Applied Biosystems). Cycling conditions for all primer pairs consisted of an initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 56°C for 15 s and 60°C for 1 min. PCR was performed in triplicates and specific annealing of the primers was controlled by monitoring dissociation kinetics at the end of each PCR run. The geometric mean of the expression ratios of two endogenous housekeeping genes (actin and ubiquitin; *ACT1, UBI* primers described in Table S2) was used as normalization factor for all samples. The efficiency of each primer pair was measured on a serial dilution of a calibrator sample (WT IRR-R). The qRT-PCR data of all tissues and genes were expressed as abundance relative to endogenous *VvPIP2;4N* in wild-type irrigated roots (IRR-R).

Measurements of physiological parameters and leaf ABA concentrations

Six transgenic lines with different T-DNA configurations were chosen for analysis: 4, 16, 23, 24, 28 and 33. Eight plants for each line, together with eight wild-type plants, were acclimated in soil in greenhouse conditions (Lovisolo et al., 2008a) and four plants for each line were subjected to a water stress treatment that consisted in withholding water for a 14-day period. Four further 'Nebbiolo' plants were water-stressed in the same conditions in order to compare their stomatal response to stress with wild-type 'Brachetto'.

CO_2 assimilation (P_n) and leaf transpiration (E) were recorded using the infra-red gas analyser ADC-LCPro+ system (The Analytical Development Company Ltd, www.adc-service.co.uk/) in the central hours of the day (11:00 am – 01:00 pm), without PAR limitations, by measuring three leaves per plant, originating from the central region of the shoot, and averaging measurements every 10 minutes. During measurements, air temperature was 32.9 ± 0.45 °C, vapour pressure deficit (VPD) was 24.8 ± 1.06 Pa/kPa, and incident photon flux density (PPFD) was 1210 ± 32.9 μmol m^{-2} s^{-1}. Water use efficiency (WUE) was calculated from CO_2 assimilation and transpiration rates. For each plant, at the end of gas exchange measurements, leaf water potential (Ψ_{leaf}) was measured on two transpiring leaves inserted in the central region of the shoot, using a Scholander-type pressure chamber (Soil Moisture Equipment Corp., www.soilmoisture.com). Soil and shoot water potential measurements, and foliar ABA content (one leaf per replicate plant collected at midday), were performed as previously described (Lovisolo et al., 2008a).

Root hydraulic conductance (k_{hroot}) was measured on well-watered plants by means of an HCFM-XP Hydraulic Conductance Flow Meter (Dynamax Inc., http//www.dynamax.com/). Total plant hydraulic resistance was partitioned as described by Lovisolo et al. (2007), by considering roots, shoots and leaves resistances as additive. Shoot and root weight was measured. Surface area-specific root hydraulic conductance was calculated using an average root surface area /dry weight ratio measured on eight 1 g root samples. To this aim, root surface was determined based on measurements of average root diameter (assessed in 100 random points at 10x magnification) and of volume (assessed gravimetrically; Lovisolo et al. 2008b), considering the root shape as cylindrical.

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LITERATURE CITED

Aharon R, Shahak Y, Wininger S, Bendov R, Kapulnik Y, Galili G (2003) Overexpression of a plasma membrane aquaporin in transgenic tobacco improves plant vigor under favorable growth conditions but not under drought or salt stress. Plant Cell **15:** 439-447

Baiges I, Schaffner AR, Mas A (2001) Eight cDNA encoding putative aquaporins in *Vitis* hybrid Richter-110 and their differential expression. J Exp Bot **52:** 1949-1951

Balestrini R, Mainieri D, Soragni E, Garnero L, Rollino S, Viotti A, Ottonello S, Bonfante P (2000) Differential expression of chitin synthase III and IV mRNAs in ascomata of *Tuber borchii* Vittad. Fungal Genet Biol **31:** 219-232

Barone LM, Shih C, Wasserman BP (1997) Mercury-induced conformational changes and identification of conserved surface loops in plasma membrane aquaporins from higher plants. Topology of PMIP31 from *Beta vulgaris* L. J Biol Chem **272:** 30672-30677

Barrowclough DE, Peterson CA, Steudle E (2000) Radial hydraulic conductivity along developing onion roots. J Exp Bot **51:** 547-557

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res **12:** 8711-8721

Biela A, Grote K, Otto B, Hoth S, Hedrich R, Kaldenhoff R (1999) The *Nicotiana tabacum* plasma membrane aquaporin NtAQP1 is mercury-insensitive and permeable for glycerol. Plant J **18:** 565-570

Brundrett MC, Enstone DE, Peterson CA (1988) A berberine-aniline blue fluorescent staining procedure for suberin, lignin and callose in plant tissue. Protoplasma **146**:133-142

Buckley TN, Sack L, Gilbert ME (2011) The role of bundle sheath extensions and life form in stomatal responses to leaf water status. Plant Physiol **156:** 962-973

Chaumont F, Barrieu F, Jung R, Chrispeels MJ (2000) Plasma membrane intrinsic proteins from maize cluster in two sequence subgroups with differential aquaporin activity. Plant Physiol **122:** 1025-1034

Choat B, Gambetta GA, Shackel KA, Matthews MA (2009) Vascular function in grape berries across development and its relevance to apparent hydraulic isolation. Plant Physiol **151:** 1677-1687

Chrispeels MJ, Maurel C (1994) Aquaporins: the molecular basis of facilitated water movement through living plant cells? Plant Physiol **105:** 9-13

Cochard H, Venisse JS, Barigah TS, Brunel N, Herbette S, Guilliot A, Tyree MT, Sakr S (2007) Putative role of aquaporins in variable hydraulic conductance of leaves in response to light. Plant Physiol **143:** 122-133

Cui XH, Hao FS, Chen H, Chen J, Wang XC (2008) Expression of the *Vicia faba VfPIP1* gene in *Arabidopsis thaliana* plants improves their drought resistance. J Plant Res **121:** 207-214

Cutanda-Perez MC, Ageorges A, Gomez C, Vialet S, Terrier N, Romieu C, Torregrosa L (2009) Ectopic expression of *VlmybA1* in grapevine activates a narrow set of genes involved in anthocyanin synthesis and transport. Plant Mol Biol **69:** 633-648

D'Angeli S, Altamura MM (2007) Osmotin induces cold protection in olive trees by affecting programmed cell death and cytoskeleton organization. Planta **225:** 1147-1163

Fouquet R, Leon C, Ollat N, Barrieu F (2008) Identification of grapevine aquaporins and expression analysis in developing berries. Plant Cell Rep **27:** 1541-1550

Galmès J, Pou A, Alsina MM, Tomas M, Medrano H, Flexas J (2007) Aquaporin expression in response to different water stress intensities and recovery in Richter-110 *(Vitis sp.*): relationship with ecophysiological status. Planta **226:** 671-681

Gambino G, Gribaudo I, Leopold S, Schartl A, Laimer M (2005) Molecular characterization of grapevine plants transformed with GFLV resistance genes: I. Plant Cell Rep **24:** 655-662

Gambino G, Perrone I, Carra A, Chitarra W, Boccacci P, Torello Marinoni D, Barberis M, Maghuly F, Laimer M, Gribaudo I (2010) Transgene silencing in grapevines transformed with GFLV resistance genes: analysis of variable expression of transgene, siRNAs production and cytosine methylation. Transgenic Res **19:** 17-27

Gelvin SB (2003) Agrobacterium-mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiol Mol Biol Rev **67:** 16-37

Hachez C, Zelazny E, Chaumont F (2006) Modulating the expression of aquaporin genes in planta: A key to understand their physiological functions? Biochim Biophys Acta **1758:** 1142-1156

Heckwolf M, Pater D, Hanson DT, Kaldenhoff R (2011) The *Arabidopsis thaliana* aquaporin *AtPIP1;2* is a physiologically relevant CO_2 transport facilitator. Plant J **67:** 795-804

Heinen RB, Ye Q, Chaumont F (2009) Role of aquaporins in leaf physiology. J Exp Bot **60:** 2971-2985

Hukin D, Doering-Saad C, Thomas CR, Pritchard J (2002) Sensitivity of cell hydraulic conductivity to mercury is coincident with symplasmic isolation and expression of plasmalemma aquaporin genes in growing maize roots. Planta **215:** 1047-1056

Iocco P, Franks T, Thomas MR (2001) Genetic transformation of major wine grape cultivars of *Vitis vinifera* L. Transgenic Res **10:** 105-112

Johanson U, Karlsson M, Johansson I, Gustavsson S, Sjovall S, Fraysse L, Weig AR, Kjellbom P (2001) The complete set of genes encoding major intrinsic proteins in Arabidopsis provides a framework for a new nomenclature for major intrinsic proteins in plants. Plant Physiol **126:** 1358-1369

Johansson I, Karlsson M, Shukla VK, Chrispeels MJ, Larsson C, Kjellbom P (1998) Water transport activity of the plasma membrane aquaporin PM28A is regulated by phosphorylation. Plant Cell **10:** 451-459

Kaldenhoff R, Fischer M (2006) Aquaporins in plants. Acta Physiologica **187:** 169-176

Kaldenhoff R, Grote K, Zhu JJ, Zimmermann U (1998) Significance of plasmalemma aquaporins for water-transport in *Arabidopsis thaliana*. Plant J **14:** 121-128

Kaldenhoff R, Ribas-Carbo M, Flexas J, Lovisolo C, Heckwolf M, Uehlein N (2008) Aquaporins and plant water balance. Plant Cell and Environment **31:** 658-666

Katsuhara M, Koshio K, Shibasaka M, Hayashi Y, Hayakawa T, Kasamo K (2003) Over-expression of a barley aquaporin increased the shoot/root ratio and raised salt sensitivity in transgenic rice plants. Plant Cell Physiol **44:** 1378-1383

Kumar S, Fladung M (2001) Gene stability in transgenic aspen (*Populus*). II. Molecular characterization of variable expression of transgene in wild and hybrid aspen. Planta **213:** 731-740

Li GW, Zhang MH, Cai WM, Sun WN, Su WA (2008) Characterization of *OsPIP2;7*, a water channel protein in rice. Plant Cell Physiol **49:** 1851-1858

Liakoura V, Fotelli MN, Rennenberg H, Karabourniotis G (2009) Should structure-function relations be considered separately for homobaric vs. heterobaric leaves? Am J Bot **96:** 612-619

Loveys B (1984) Abscisic acid transport and metabolism in grapevine (*Vitis vinifera L.*). New Phytologist **98:** 575-582

Lovisolo C, Perrone I, Carra A, Ferrandino A, Flexas J, Medrano H, Schubert A (2010) Drought-induced changes in development and function of grapevine (*Vitis* spp.) organs and in their hydraulic and non-hydraulic interactions at the whole-plant level: a physiological and molecular update. Funct Plant Biol **37:** 98-116

Lovisolo C, Perrone I, Hartung W, Schubert A (2008a) An abscisic acid-related reduced transpiration promotes gradual embolism repair when grapevines are rehydrated after drought. New Phytol **180:** 642-651

Lovisolo C, Secchi F, Nardini A, Salleo S, Buffa R, Schubert A (2007) Expression of PIP1 and PIP2 aquaporins is enhanced in olive dwarf genotypes and is related to root and leaf hydraulic conductance. Physiol Plant **130:** 543-551

Lovisolo C, Tramontini S, Flexas J, Schubert A (2008b) Mercurial inhibition of root hydraulic conductance in *Vitis* spp. rootstocks under water stress. Environ Exp Bot **63:** 178-182

Maghuly F, Leopold S, da Camara Machado A, Borroto Fernandez E, Ali Khan M, Gambino G, Gribaudo I, Schartl A, Laimer M (2006) Molecular characterization of grapevine plants transformed with GFLV resistance genes: II. Plant Cell Rep **25:** 546-553

Marjanovic Z, Uehlein N, Kaldenhoff R, Zwiazek JJ, Weiss M, Hampp R, Nehls U (2005) Aquaporins in poplar: what a difference a symbiont makes! Planta **222:** 258-268

Martre P, Morillon R, Barrieu F, North GB, Nobel PS, Chrispeels MJ (2002) Plasma membrane aquaporins play a significant role during recovery from water deficit. Plant Physiol **130:** 2101-2110

Matsumoto T, Lian HL, Su WA, Tanaka D, Liu C, Iwasaki I, Kitagawa Y (2009) Role of the aquaporin PIP1 subfamily in the chilling tolerance of rice. Plant Cell Physiol **50:** 216-229

Maurel C, Verdoucq L, Luu DT, Santoni V (2008) Plant aquaporins: membrane channels with multiple integrated functions. Ann Rev of Plant Biol **59:** 595-624

Moshelion M, Becker D, Biela A, Uehlein N, Hedrich R, Otto B, Levi H, Moran N, Kaldenhoff R (2002) Plasma membrane aquaporins in the motor cells of *Samanea saman*: diurnal and circadian regulation. Plant Cell **14:** 727-739

Nardini A, Gortan E, Salleo S (2005) Hydraulic efficiency of the leaf venation system in sun- and shade-adapted species. Funct Plant Biol **32:** 953-961

North GB, Martre P, Nobel PS (2004) Aquaporins account for variations in hydraulic conductance for metabolically active root regions of Agave deserti in wet, dry, and rewetted soil. Plant Cell Environ **27:** 219-228

Peng Y, Arora R, Li G, Wang X, Fessehaie A (2008) *Rhododendron catawbiense* plasma membrane intrinsic proteins are aquaporins, and their over-expression compromises constitutive freezing tolerance and cold acclimation ability of transgenic *Arabidopsis* plants. Plant Cell Environ **31:** 1275-1289

Picaud S, F. B, Dédaldécheamp F, Ageorges A, Delrot S (2003) Cloning and expression of two plasma membrane aquaporins expressed during the ripening of grape berry. Funct Plant Biol **30:** 621-630

Postaire O, Tournaire-Roux C, Grondin A, Boursiac Y, Morillon R, Schaffner AR, Maurel C (2010) A PIP1 aquaporin contributes to hydrostatic pressure-induced water transport in both the root and rosette of *Arabidopsis*. Plant Physiol **152:** 1418-1430

Reustle GM, Büchholz G (2009) Recent trends in grapevine genetic engineering. *In* KA Roubelakis-Angelakis, ed, Grapevine Molecular Physiology & Biotechnology, Vol 495-508. Springer Netherlands Publ., Dordrecht

Sade N, Vinocur BJ, Diber A, Shatil A, Ronen G, Nissan H, Wallach R, Karchi H, Moshelion M (2009) Improving plant stress tolerance and yield production: is the tonoplast aquaporin SlTIP2;2 a key to isohydric to anisohydric conversion? New Phytol **181:** 651-661

Sakurai J, Ishikawa F, Yamaguchi T, Uemura M, Maeshima M (2005) Identification of 33 rice aquaporin genes and analysis of their expression and function. Plant Cell Physiol **46:** 1568-1577

Secchi F, Lovisolo C, Uehlein N, Kaldenhoff R, Schubert A (2007) Isolation and functional characterization of three aquaporins from olive (*Olea europaea* L.). Planta **225:** 381-392

Secchi F, Zwieniecki MA (2010) Patterns of PIP gene expression in *Populus trichocarpa* during recovery from xylem embolism suggest a major role for the PIP1 aquaporin subfamily as moderators of refilling process. Plant Cell Environ **33:** 1285-1297

Shelden MC, Howitt SM, Kaiser BN, Tyerman SD (2009) Identification and functional characterisation of aquaporins in the grapevine, *Vitis vinifera*. Funct Plant Biol **36:** 1065-1078

Siefritz F, Tyree MT, Lovisolo C, Schubert A, Kaldenhoff R (2002) PIP1 plasma membrane aquaporins in tobacco: From cellular effects to function in plants. Plant Cell **14:** 869-876

Sorieul M, Santoni V, Maurel C, Luu DT (2011) Mechanisms and effects of retention of over-expressed aquaporin AtPIP2;1 in the endoplasmic reticulum. Traffic **12:** 473-482

Stoll M, Loveys B, Dry P (2000) Hormonal changes induced by partial rootzone drying of irrigated grapevine. J Exp Bot **51:** 1627-1634

Tesnière C, Torregrosa L, Pradal M, Souquet JM, Gilles C, Dos Santos K, Chatelet P, Gunata Z (2006) Effects of genetic manipulation of alcohol dehydrogenase levels on the response to stress and the synthesis of secondary metabolites in grapevine leaves. J Exp Bot **57:** 91-99

Thomas MR, Matsumoto S, Cain P, Scott NS (1993) Repetitive DNA of grapevine: classes present and sequences suitable for cultivar identification. Theor. Appl. Genet. **86:** 173-180

Tournaire-Roux C, Sutka M, Javot H, Gout E, Gerbeau P, Luu DT, Bligny R, Maurel C (2003) Cytosolic pH regulates root water transport during anoxic stress through gating of aquaporins. Nature **425:** 393-397

Tsuchihira A, Hanba YT, Kato N, Doi T, Kawazu T, Maeshima M (2010) Effect of overexpression of radish plasma membrane aquaporins on water-use efficiency, photosynthesis and growth of *Eucalyptus* trees. Tree Physiol **30:** 417-430

Tyree MT, Nardini A, Salleo S, Sack L, El Omari B (2005) The dependence of leaf hydraulic conductance on irradiance during HPFM measurements: any role for stomatal response? J Exp Bot **56:** 737-744

Vandeleur RK, Mayo G, Shelden MC, Gilliham M, Kaiser BN, Tyerman SD (2009) The role of plasma membrane intrinsic protein aquaporins in water transport through roots: diurnal and drought stress responses reveal different strategies between isohydric and anisohydric cultivars of grapevine. Plant Physiol **149:** 445-460

Yang YZ, Costa A, Leonhardt N, Siegel RS, Schroeder JI (2008) Isolation of a strong *Arabidopsis* guard cell promoter and its potential as a research tool. Plant Methods **4:** 6

Zelazny E, Miecielica U, Borst JW, Hemminga MA, Chaumont F (2009) An N-terminal diacidic motif is required for the trafficking of maize aquaporins ZmPIP2;4 and ZmPIP2;5 to the plasma membrane. Plant J **57:** 346-355

Zhang Y, Wang Z, Chai T, Wen Z, Zhang H (2008) Indian mustard aquaporin improves drought and heavy-metal resistance in tobacco. Mol Biotechnol **40:** 280-292

FIGURE LEGENDS

Figure 1. Neighbour-joining tree of grape and Arabidopsis PIP proteins. Protein sequences of Genbank accessions for *V. vinifera* (cv Pinot noir, Cabernet Sauvignon, Nebbiolo), for *Vitis berlandieri* x *Vitis rupestris*, and for *Arabidopsis thaliana*, and of genomic loci from the *V. vinifera* (cv PN40024) 12X genome draft (http://genomes.cribi.unipd.it/) were clustered using Mega4. The significance of each node was tested using 1000 bootstrap replicates.

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SUPPLEMENTAL DATA

Additional Supplemental Data can be found in the online version of this article:

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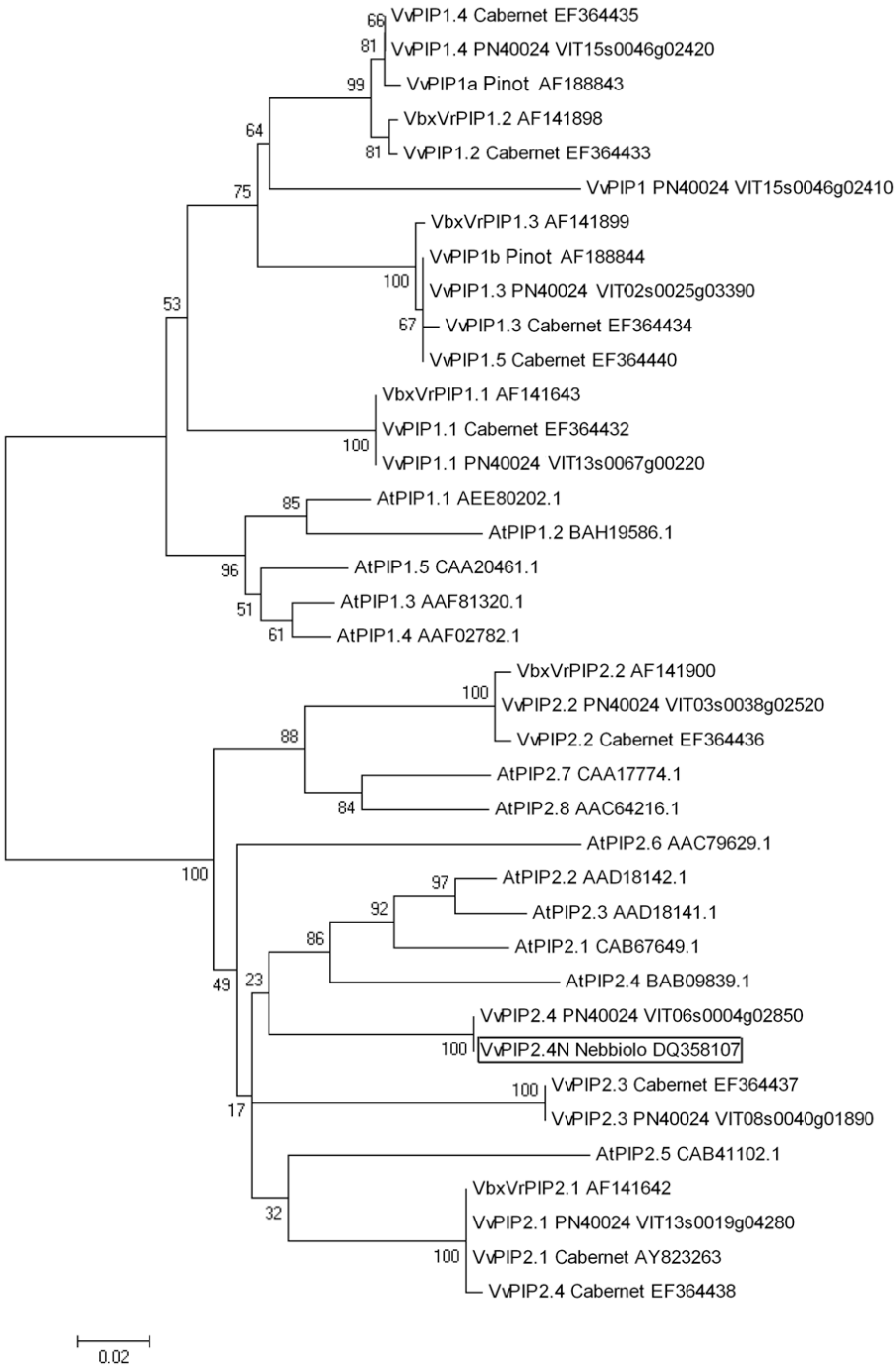

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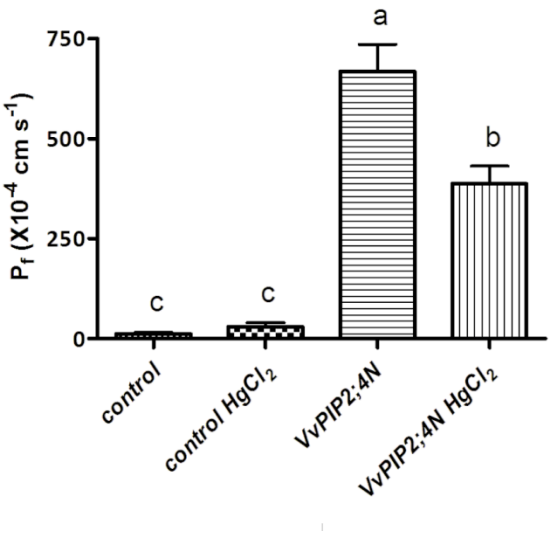

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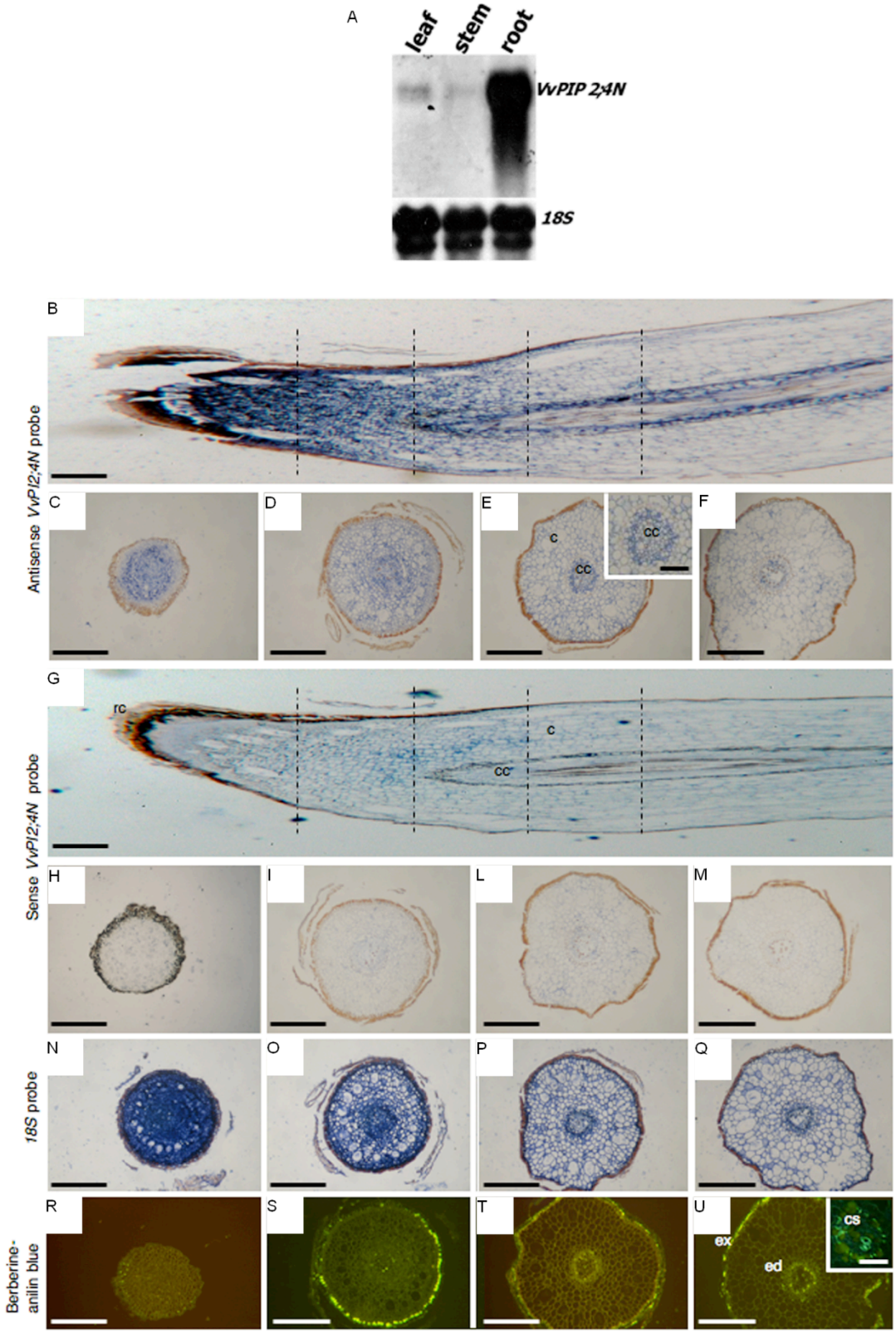
A
leaf
stem
root
VvPIP 2;4N
18S
B
Antisense VvPI2;4N probe
C
D
E
c
cc
cc
F
G
Sense VvPI2;4N probe
rc
c
cc
H
I
L
M
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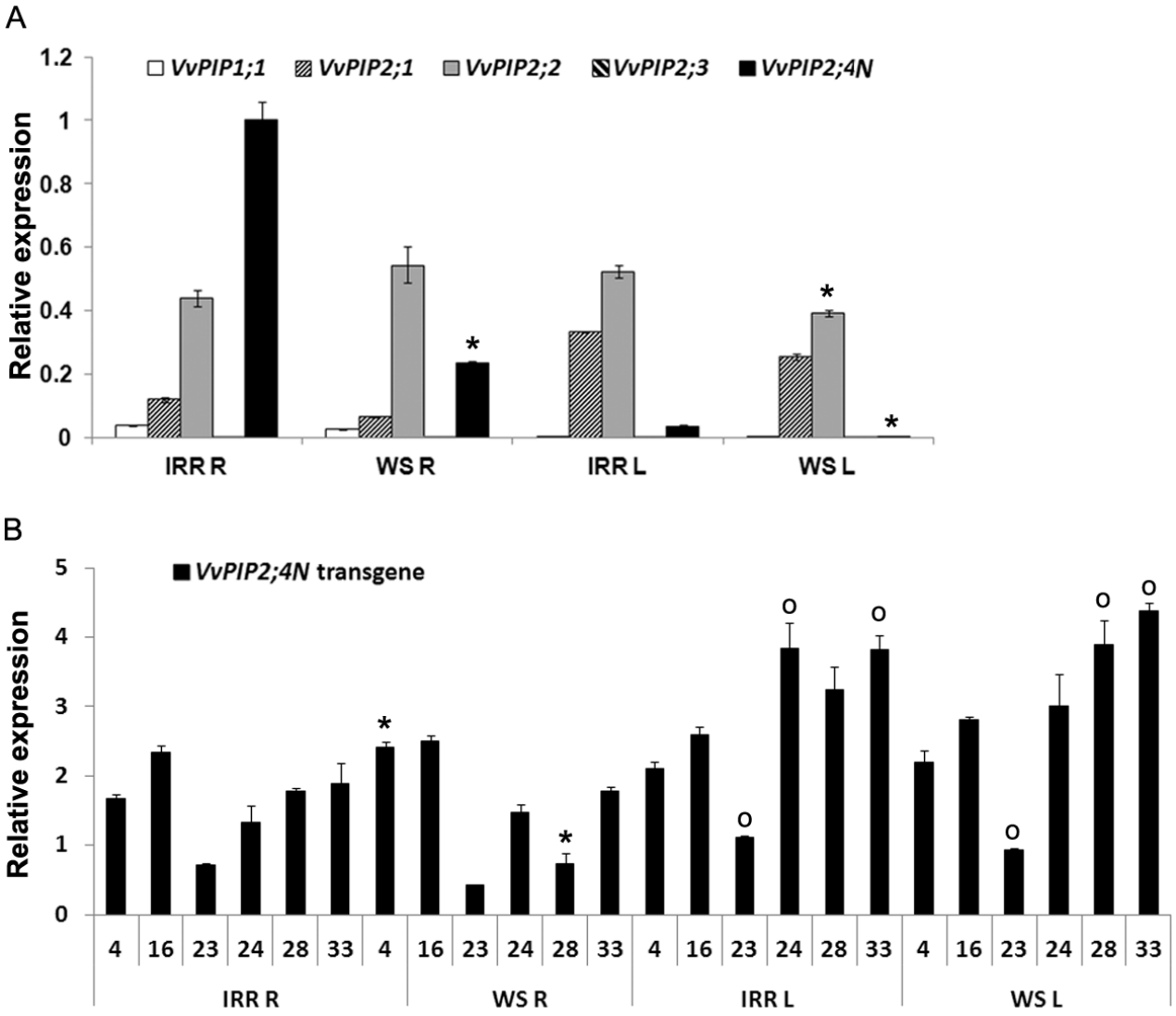

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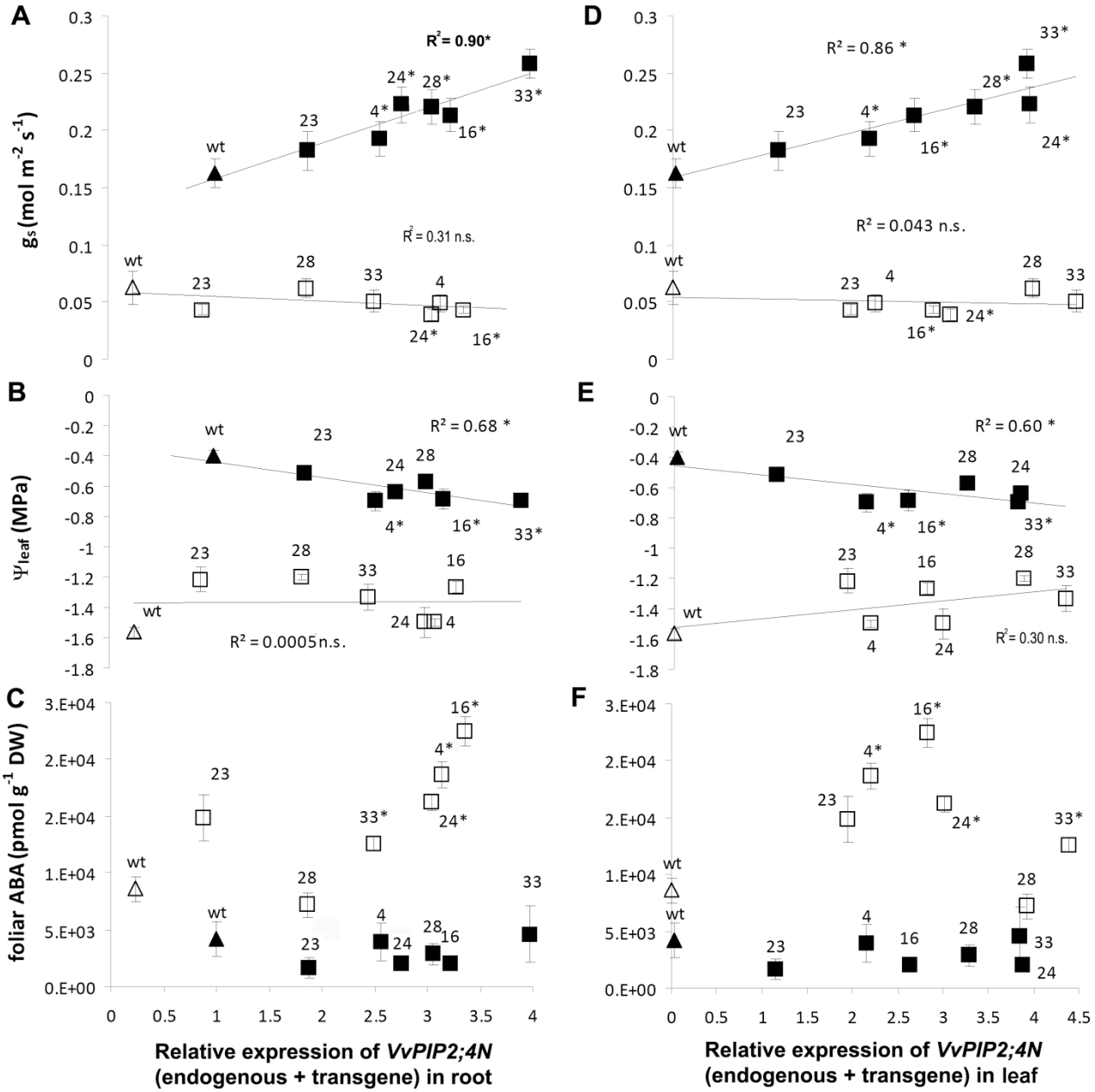

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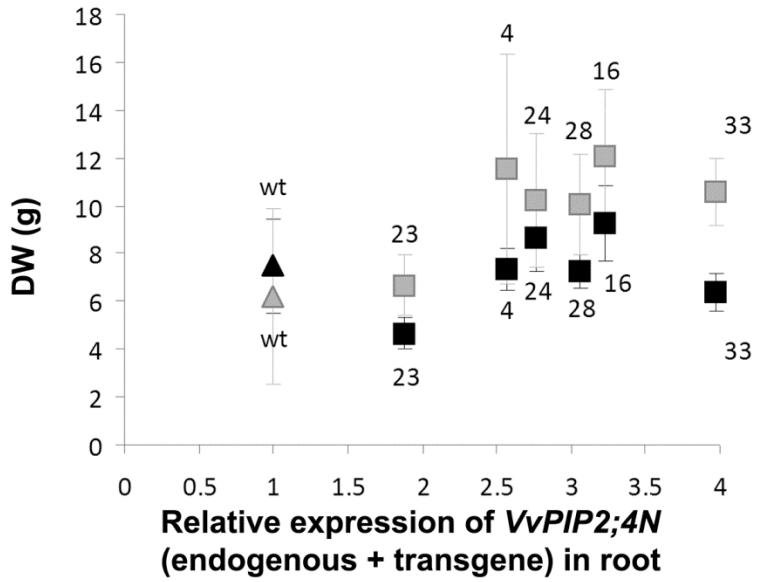

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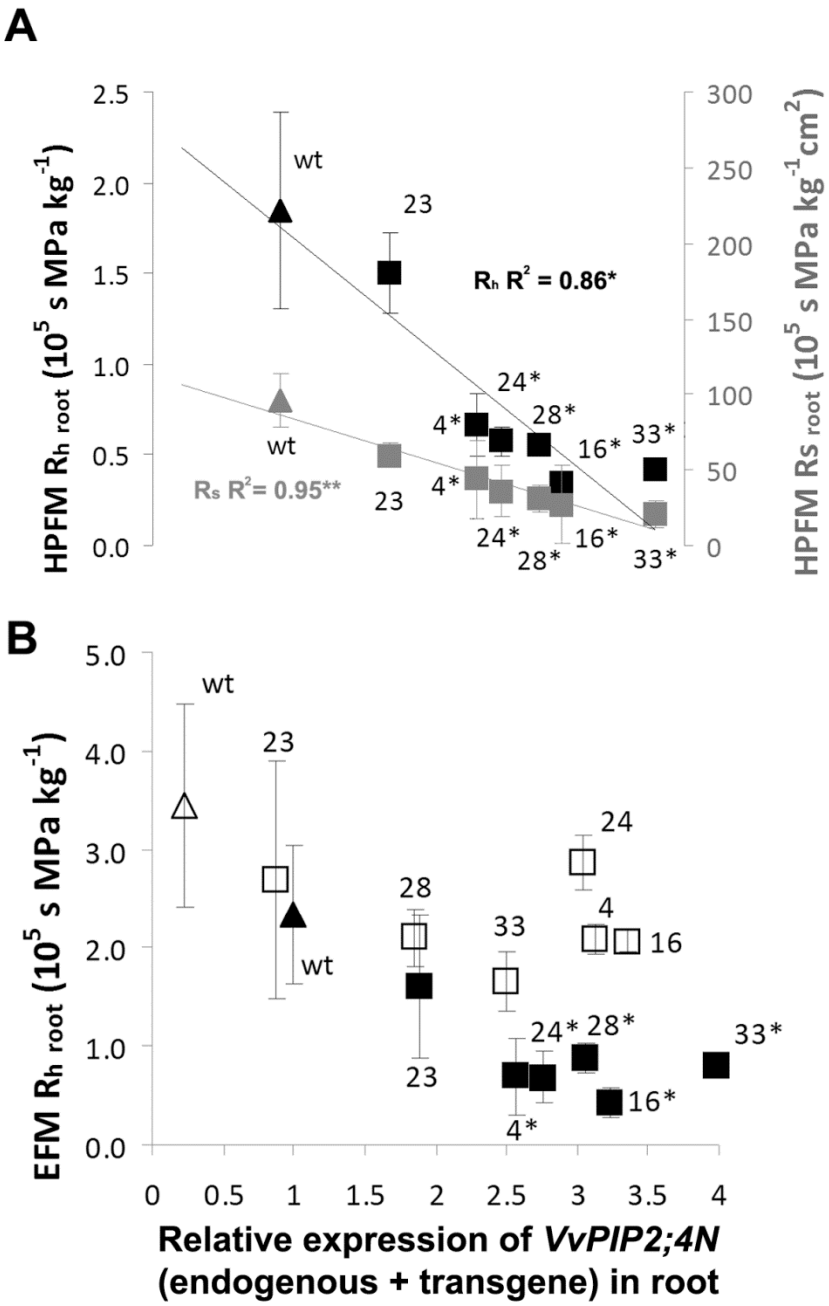

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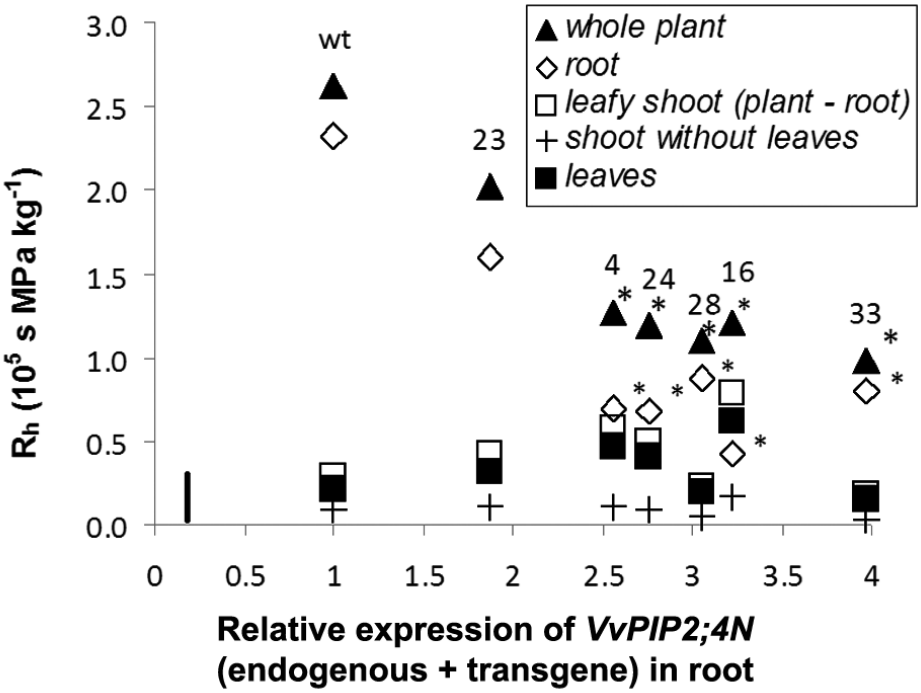

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Table S1**.** Transgenic 'Brachetto' lines showing the same hybridization pattern in Southern blots. Lines used in the physiological analyses are underlined.

T-DNA copy number	Transgenic lines with the same hybridization pattern
1	10 / 12 / 19 / 22 / 26
1	13 / 21
1	16 / 18
1	4
2	1 / 2 / 25 / 29
2	7 / 9 / 14 / 15 / 33 / 34
2	8 / 11 / 20 / 30
2	5 / 17 / 31
2	23
3/4	28
5/6	3 / 6 / 24 / 27

Table S2. Oligonucleotides used in this study.

Gene	GenBank accession number	Primer Sequences 5'-3'		Application[a]
VvPIP2;4N	DQ358107	For	CGGGATCCCGCGGTGGTAAACAATGACGAAAGACG	1
		Rev	CGGGATCCCGCCAAAACCTAGGCATTGCTCCTG	
		For	CGGGATCCCGCGGTGGTAAACAATGACGAAAGACG	2
		Rev	AAGGAATTCCCCAAAACCTAGGCATTGCTCCTG	
		For	CTTCGCAAGACCCTTCGTCT	3
		Rev	CGGGATCCCGCCAAAACCTAGGCATTGCTCCTG	
		For	CTAGGATCTTTCAGGAGCAA	4
		Rev	TACTCCTCCACCATTGATGT	
		For*	CTGCCATTGCTGCATTCTACCA	5
		Rev*	TGGTGATTTCAGCGTACCGAATTC	
VvPIP2;1	AY823263	For #	CCATTTTGATACCTTCTTCC	4
		Rev#	TATCTACAATTTCATGCCCTC	
VvPIP2;2	EF364436	For#	AACTAAAAACCCACAACACCC	
		Rev#	CATCATCATAATCATCTCTGG	
VvPIP2;3	EF364437	For#	CATTTCAATCCACATGGTCCG	
		Rev#	CCACAAATTCGTCACACATCC	
VvPIP1;1	EF364432	For#	GAGTGGTGCTGGGCGTTGATC	
		Rev#	GTGGAATGCTACAGACATTAC	
VvACT1	XM_002282480	For	GCCCCTCGTCTGTGACAATG	
		Rev	CCTTGGCCGACCCACAATA	
VvUBI	XM_002273532	For	TCTGAGGCTTCGTGGTGGTA	
		Rev	AGGCGTGCATAACATTTGCG	

[a]:

1: primers containing *Bam*HI restriction sites used for cloning *VvPIP2;4N* into the pGEM-T Easy vector (Promega), into the binary vector pJam1469 for *A. tumefaciens*-mediated transformation, and for the synthesis of the digoxigenin labelled probes used for Northern blot analysis and for *in situ* hybridization

2: primers containing *Bam*HI (For) and *Eco*RI (Rev) restriction sites used for cloning *VvPIP2;4N* in the expression vector for the functional assay in *X. laevis* oocytes

3: primers used to detect transgenic *VvPIP2;4N* in the genome of transformed plants (forward primer designed on 35S promoter)

4: primers used for qRT-PCR quantification of transcripts of endogenous genes (reverse primer for *VvPIP2;4N* designed on 3'-UTR); # = primers designed by Choat et al. (2009)

5: primers used for qRT-PCR quantification of transgenic *VvPIP2;4N* (reverse primer designed on terminator)

Legends to Supplementary Figures

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Figure S2. Southern analysis of DNA from 'Nebbiolo' and 'Brachetto'. A, DNA from 'Nebbiolo' was digested with XbaI, and DIG-labelled full-length *VvPIP2;4N* was used as probe. A single band was detected, indicating hybridization to a single locus. B and C, DNA from 'Brachetto' lines was digested with EcoRI, and DIG-labelled *nptII* was used as probe. The control DNA (wt) is from an untransformed 'Brachetto' plant.

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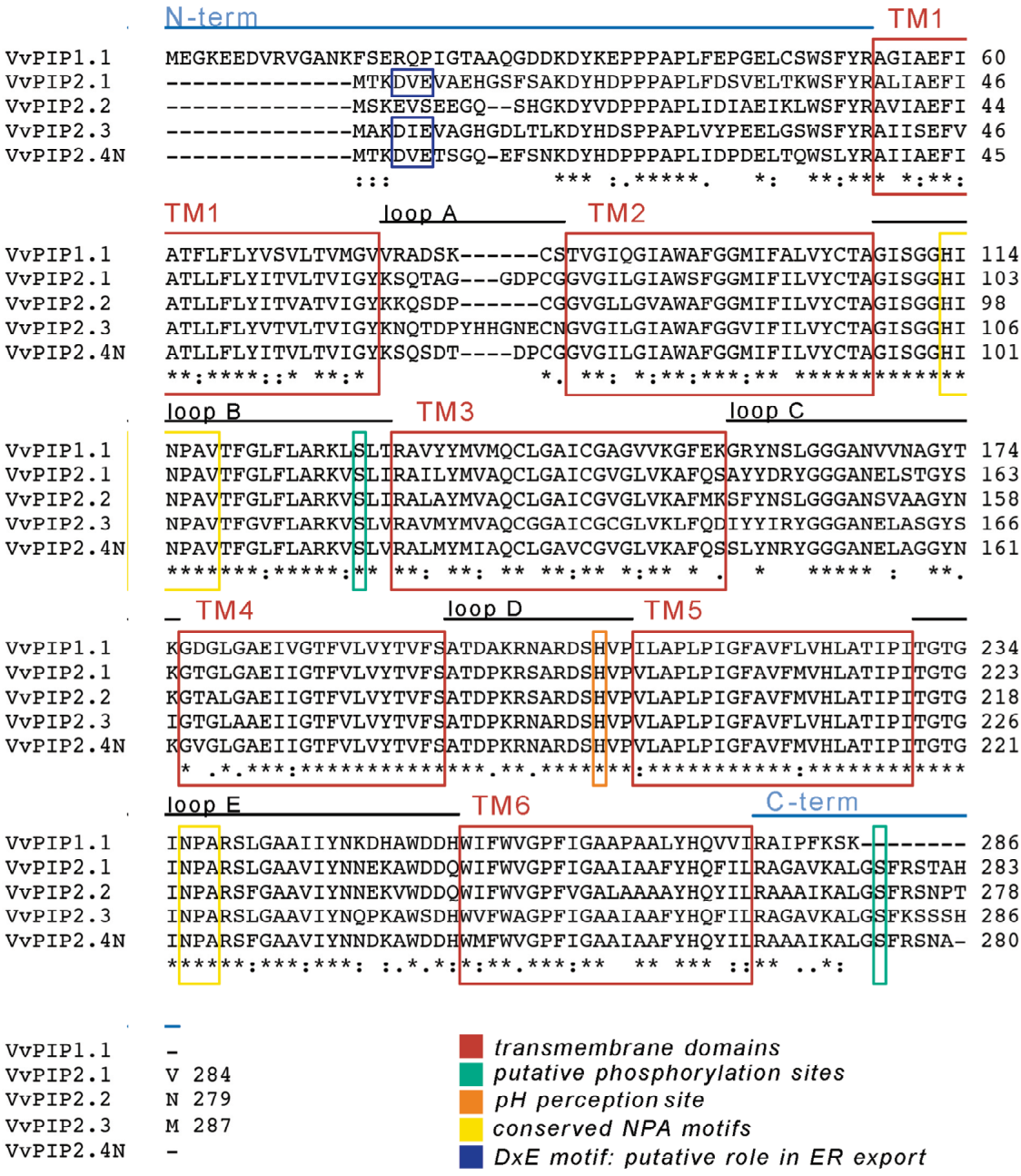
Figure S5. Relationship between stomatal conductance (g_s) and leaf water potential (Ψ_{leaf}) in pot-grown 'Brachetto' and 'Nebbiolo' plants subjected to different levels of water stress (means ± SE; n=8).



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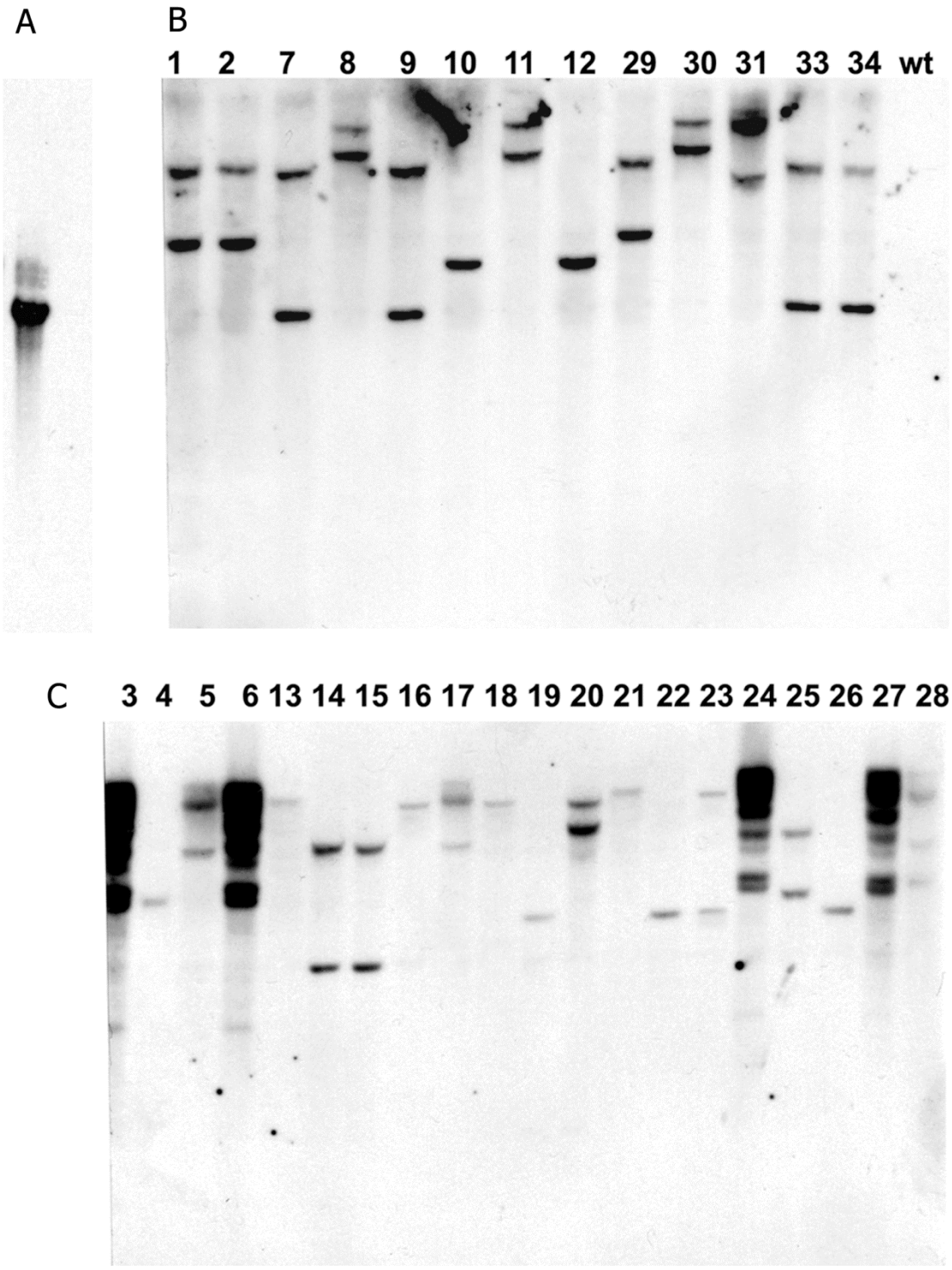

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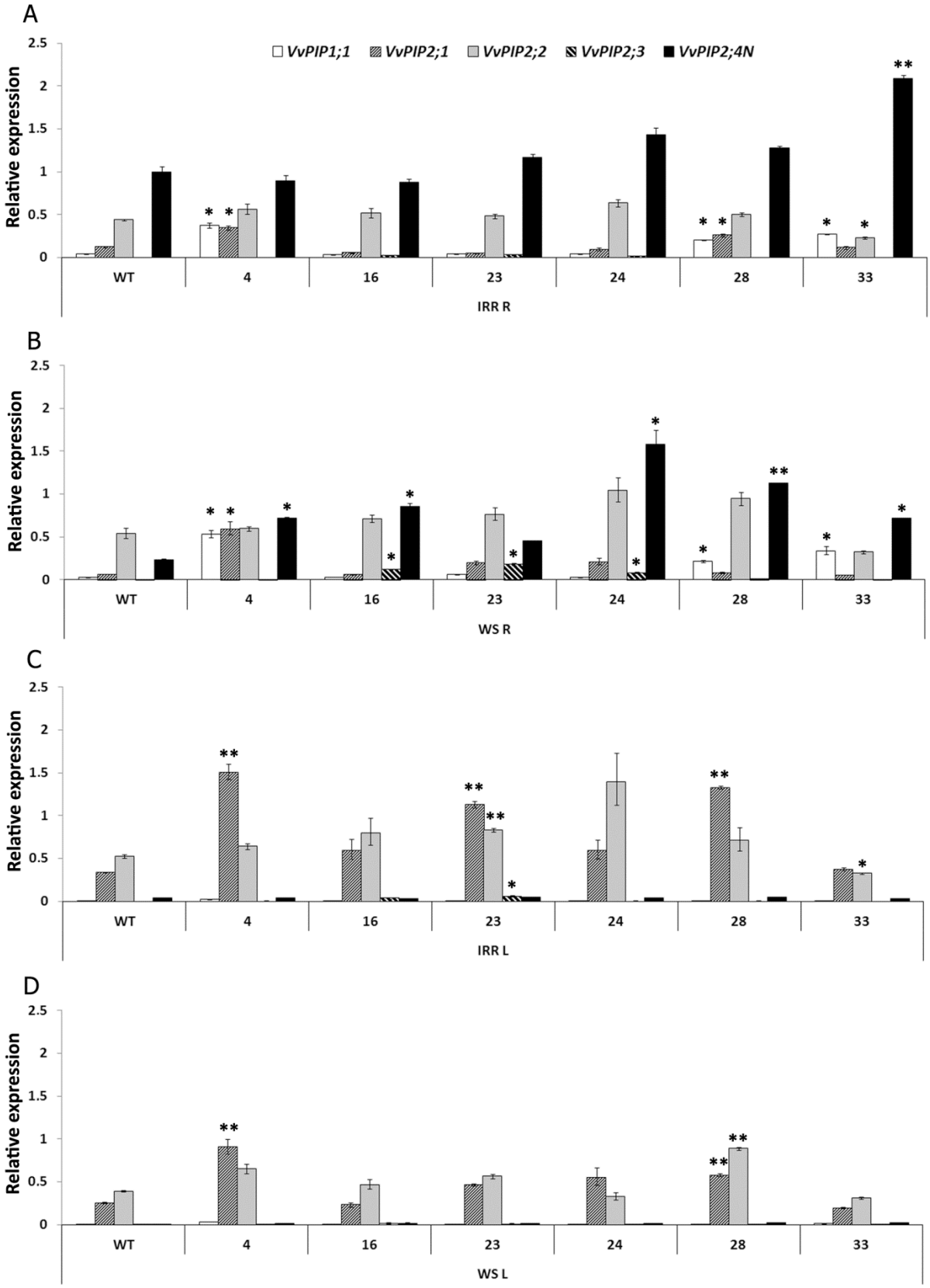

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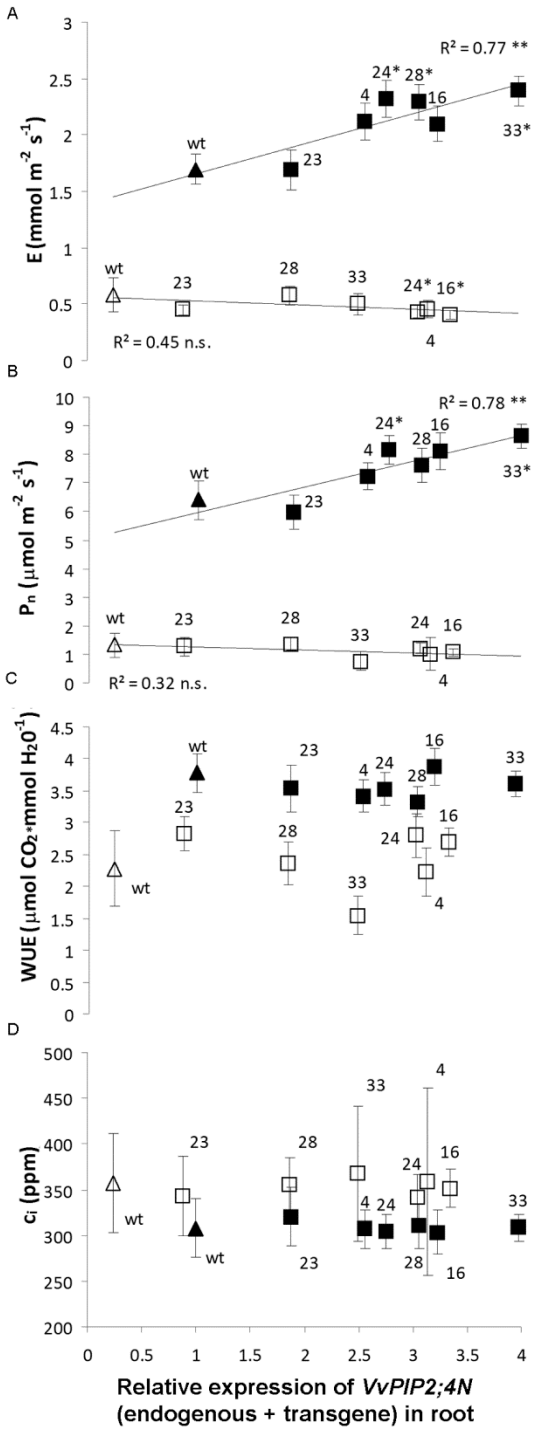

Figure S4. Leaf gas exchanges in transgenic 'Brachetto' plants. A, Leaf transpiration (E); B, net photosynthesis (P_n); C, water use efficiency (WUE), and (D) calculated CO_2 concentration in substomatal cavities (C_i) were assessed in wild-type (triangles) and transgenic plants (squares) belonging to six lines (4, 16, 23, 24, 28, 33) upon well-watered (filled symbols) and water stress (empty symbols) conditions (means ± SE; n=8). Data are plotted in dependence of *VvPIP2;4N* expression in root. Asterisks mark significant differences to

wild-types, assessed with Student t test, and significance of regression to root expression levels of *VvPIP2,4N* (endogenous + transgene) (* $P<0.05$; ** $P<0.01$).

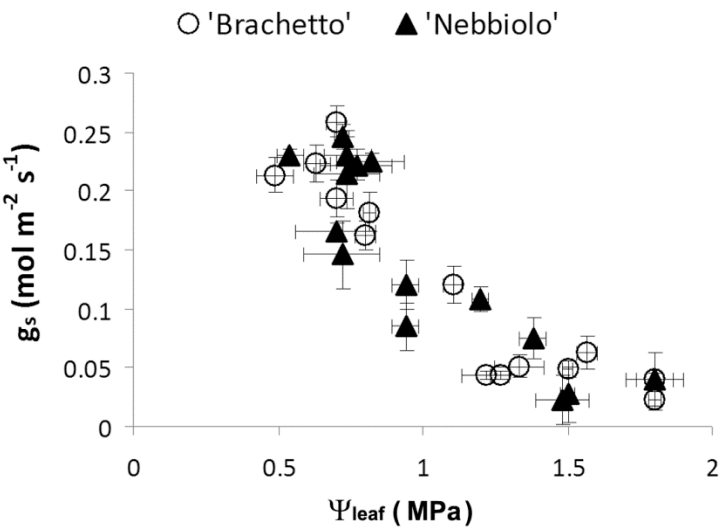

Figure S5. Relationship between stomatal conductance (g_s) and leaf water potential (Ψ_{leaf}) in pot-grown 'Brachetto' and 'Nebbiolo' plants subjected to different levels of water stress (means ± SE; n=8).